رقم البحث (6)

EXPERIMENTAL PATHOGENICITY OF TWO SAPROLEGNIA SPP. TO NILE TILAPIA (Oreochromis niloticus) IN EGYPT, WITH EMPHASIS ON HISTOPATHOLOGICAL ALTERATIONS

BY

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ABSTRACT

Saprolegniosis is one of the important mycotic diseases affecting fish worldwide and causing huge economic losses. Here, we evaluate the Pathogenicity and pathology of two important isolates, *Saprolegnia parasitica* and *Saprolegnia ferax* isolated from natural outbreaks of saprolegniosis in different fish farms in Egypt. Nile tilapia (*Oreochromis niloticus*) was challenged with two *Saprolegnia* isolates; *S. parasitica* and *S. ferax* at a zoospore concentration of $2x10^4$/L for 10 days. The cumulative mortalities were 95.6% and 70% of *S. parasitica* and *S. ferax*, respectively. The histopathological lesions of the saprolegniosis varied from vacuolation, erosion, ulceration to complete loss of the epidermis. Dermis showed edema, congestion with aggregation of inflammatory cells around hyphae and spores. Several degenerative and necrotic changes were observed in the underlying musculature. Our results confirmed not only the pathogenicity of the both isolates, but also, emphasize that *S. parasitica* is more pathogenic to Nile tilapia than *S. ferax*.

Keywords: Nile tilapia, Saprolegniosis, Tissue alteration; and Mortality

INTRODUCTION

Fish pathogenic Oomycetes, especially *Saprolegnia* spp; a member of the family Saprolegniaceae, causing major outbreaks in freshwater fish and fish hatcheries as well; these infections are widespread and occurred at any stage of fish life cycle **(Shaheen, 1986;**

Hussein et al., 2000). Saprolegniosis characterized by a relatively superficial, cottony/woolly (floccous), white growth on the injured skin, or gills, or on fish eggs when in water. Initial lesions are often focal, small, and inconspicuous, but these can rapidly enlarge because of the rapid development of the mycelium over a short period of time. Lesions may extend into dermis and the subjacent superficial musculature with time with sloughing of the epidermis **(Van West, 2006).**

Many stressors such as adverse water temperature, poor water quality, handling, or crowding are frequently associated with outbreaks of saprolegniosis (**Whisler, 1996)**. Epithelial damage on the skin and gills due to trauma or other pathogens, can provide a route of entry for Oomycetes (**Roberts, 2001).** Signs of disease and mortalities were culmination of two related factors: (I) rapid decreases in water temperature from ~20 to 10^{o}C in 24 h induced subsequent immunosupprcssion; and (2) maintenance of low temperatures favoured high levels of *Saprolegnia* sp., zoospores (≥5 spores ml**) (Bly et al., 1992).**

Fish epidermis is in direct contact with the environment and mucus is considered to be a first line of defence protecting the epidermis. In fish, the epidermis represents an initial site for complex immune responses against waterborne pathogens **(Kearn, 1999**).Rapid decrease in water temperature results in the loss of protective mucus from the epidermis and increases susceptibility to saprolegniosis. Thus, temperature is an important factor in the development of water mould infection. Most epidemics occur when temperatures are low that affect mucous cell distribution in the epidermis.

Epidermal damage facilitates the entry of the pathogen rather than producing osmo-regulatory disturbances which lead to death in severely affected cases **(Noga, 2000)**. Healing and regeneration of tissue damage involve two component factors; physiological and immunological ones **(Medzhitov, 2008).** Thus, the regeneration process of the epidermis acts a vital role in protecting against external invaders (**Quilhac and Sire, 1999; Bockelmann, 2010)**

In Egypt, fish farms at winter suffered from signs of fungal infection causing severe damage affecting eggs hatchability and evoke higher mortalities. The present study aimed to evaluate the pathogenicity of two different isolates of *Saprolegnia* spp., on Nile tilapia (*Oreochromis niloticus*) and their pathological effect.

MATERIALS AND METHODS

Naturally infected fish

Eighteen *O. niloticus* fish were collected from fish farm in Manzala, Dakhlyia, Egypt, at the period between December- February, 2010-2011. Fish were transferred to fish diseases Lab faculty of veterinary medicine, Mansoura University. Diseased fish showed heavy saprolegnia infection on fish body, head and fins wet mount preparations were directly examined

Experimental fish

Fifteen Nile tilapia (*O. niloticus*) apparently healthy were obtained from Private fish farm in Gamasa, Dakahliya, Egypt. Nile tilapia were placed to a five 60-l aquaria ran in duplicate to each group as follows: group 1 were used for exposure to zoospores of *S. ferax*; group 2 were used for exposure to zoospores of *S. parasitica*; and control group was used as a negative control. *Saprolegnia*-challenged group, were de-scaled on different regions on the body using sharp scalpel then zoospores suspension of concentration (2 x10^4 spore/L) were added to group 1 and 2 immediately. For the sham-challenged control group, they were descaled too but no zoospores were added. Fish fed on commercial diet close to satiation and maintained on 12 h light/ 12 h dark photoperiod. Water quality during all experiments was: dissolved oxygen 6.8–7.5 mg/l, temperature 15^{o}C, pH 6.65–6.87, unionized ammonia <0.001 mg/l and nitrite <0.10 mg/l. fish were acclimated for 2 weeks. All aquaria were covered during the tests to minimize contamination. Fish with zoospores exposures lasted for 3days post zoospores exposure fish were transferred to new aquaria at 15^{o}C. The aquaria were checked each day after the challenge (defined as day 0) for 10 days, and dead and moribund fish were removed for examination. All remaining fish remaining at the end of the 10-day period all remaining fish were subjected for examination. Skin scrapings and gill and fin biopsies were examined; water mould infection was confirmed via identification of broad aseptate hyphae, sporangia and encysted zoospores with light microscopy.

Fungal isolates

Two *Saprolegnia* isolates were used in this study; *S. ferax* and *S. parasitica*. These isolates were isolated from skin lesions of Nile tilapia suffered saprolegniosis from different fish farms in Egypt. Isolates were identified according to their morphological and sexual

character and sequenced (unpublished data). Fungal isolates were cultured on glucose yeast extract (GY) agar at 19°C. Agar with mycelia was then aseptically cut into 1x1cm^2 squares and placed into a Petri dish with 30 mL GY broth. After 2 days, the agar remnants were removed, and the growing mycelia were cut and washed repeatedly in sterilized tap water (TW) and then transferred into 20 mL fresh sterilized TW and kept for 18–24 h at 19°C **(Kitancharoen and Hatai, 1996**). After the zoospores of the tested *Saprolegnia* isolates were harvested; they were counted using haemocytometer and then added to experimental tanks at a concentration of 2x10^4 zoospore/l.

Fungal identification

Saprolegnia infection was identified by their morphological characters on Nile tilapia by direct microscopic examination of lesion; hyphal growth wet mount preparation, slides stained with lactophenol cotton blue, culture on glucose yeast extract (GY) agar at 19°C and inoculation on hemp seed for Sexual reproduction. Identification was based on the classical morphological criteria of **Willoughby (1978, 1985) and Johnson et al. (2002)**

Histopathological examination

Tissue specimens from moribund and dead fish were taken from skin (10x10mm; immediately under the dorsal fin) and from caudal fin (1 x1 cm; anterio-ventral portion) excised from a similar site on each fish. Other specimens were taken from gills. All specimens were subsequently fixed in 10% neutral buffered formalin, embedded in paraffin wax. Serial sections were cut from each block at 5u thickness. One set of the serial was stained with H&E and the other set was prepared with 1% periodic acid for 10 min, thoroughly rinsed with running tap water followed by incubation in Schiff's reagent for 20 min. After rinsing in tap water, the sections were counterstained with Mayer's haematoxylin **(Roberts, 2001).** All slides were examined using light microscopy.

RESULTS

Isolation and identification

Isolates of *S. parasitica* and *S. ferax* from naturally infected fish showed the branched aseptated hyphae in wet mount **(Fig. 3)** Sexual characters on hemp seed showing the oogonia with the oospores were identified **(Fig. 4, 5).**

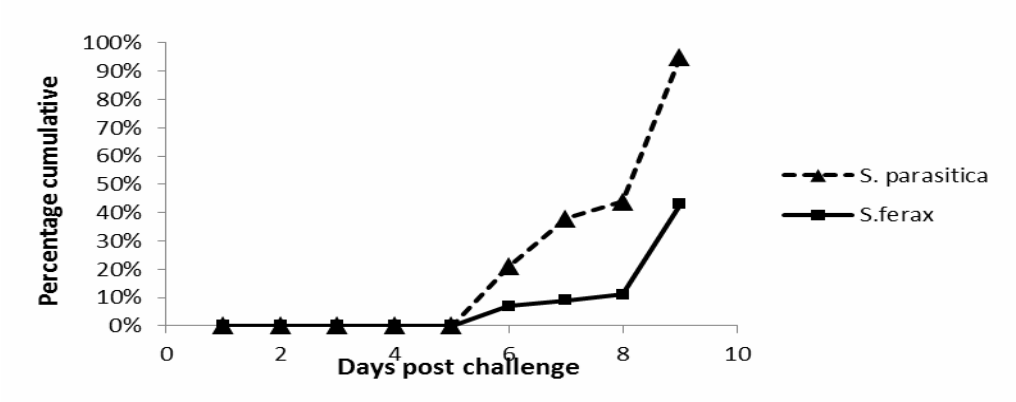
Pathogenicity experiment

Higher mortalities were evidenced between the two groups exposed to $2x10^4$ spore/L. The cumulative mortalities tend to be higher with *S. parasitica* group (95.5%) than *S. ferax* exposed group (70%). Water mold infection was first grossly visible on Day 5 post-challenge with higher mortality (21%) in *S. parasitica* and lower percent (7%) in *S. ferax* group. Mortalities continued to be at a higher rate in *S. parasitica* group reaching its peak by Day 7 (44%) while in *S. ferax* group continued but at a lower rate than *S. parasitica* and reached its peak by day 8 (43%). In contrast, all of the fish in the negative control group survived to the end of the experiments without manifestation of the characteristic external clinical signs of saprolegniosis **(Fig. 1).** The affected fish had typical signs of water mould infection, with cotton-like growths on the body and fins associated with listlessness, erratic swimming, and rising near water surfaces or resting with their abdomen on the aquarium. All dead fish showed fungal growth on body surface in particular, head, dorsal and caudal fins **(Figs. 2)**

Histopathology

Control fish displayed the normal structure of skin and underlying musculature **(Fig. 6).** Generally, the histopathological changes of the saprolegniosis were found in all sites of infection; skin showed vacuolar degeneration, erosion and ulceration **(Figs. 7&8)**. In most severe cases, the whole thickness of epidermis was lost. Congestion, edema and haemorrhage were detected in the underlying dermis. Aggregation of inflammatory cells mainly round cells was seen in superficial layer of dermis **(Figs. 8&9)**. The underlying muscles exhibited intramuscular edema, hyaline degeneration and Zenker‘s necrosis. The necrotic muscles infiltrated with numerous mononuclear leukocytes and some melanomacrophages **(Fig. 10)**. *Saprolegnia spp*, appeared by H&E stain as basophilic aseptate hyphae of variable sizes and lengths inside muscular tissue surrounded by round cells **(Fig. 11)**. PAS stain was able to detect hyphae in subcutaneous muscle **(Fig. 12)**. The histopathological changes in the affected fins included epidermal degeneration, erosion, and ulceration associated with hypodermal edema **(Figs. 13&14)**. The gills showed epithelial hyperplasia and fusion of the majority of the secondary lamellae with congestion and few mononuclear leukocytes infiltrated the primary lamellae. The gill arch was edematous. Mucous metaplasia of epithelial covering gill arch with eosinophils and melanomacrophages infiltration was noticed as well.

Fig.1. Cumulative mortalities of two saprolegnia isolates in Nile tilapia (*O.niloticus*) exposed to $2x10^4$ zoospores suspension



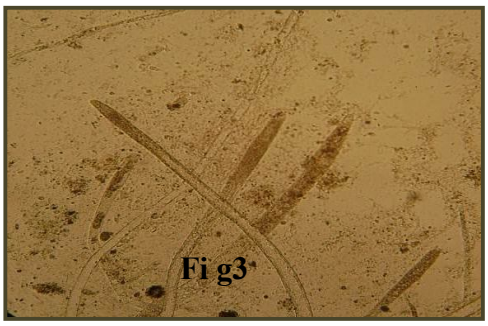
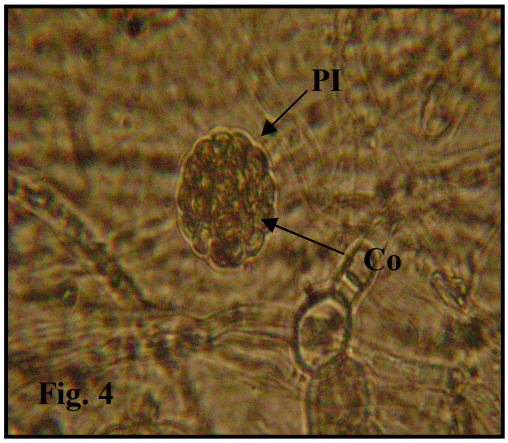


Fig.2. Nile tilapia infected with *saprolegnia* isolates with its characteristic signs of cottony wool like mycelia masses on different parts of the body.

Fig.3. wet mount preparation of the mycelial growth showing branched aseptated hyphae with apical zoosporangia



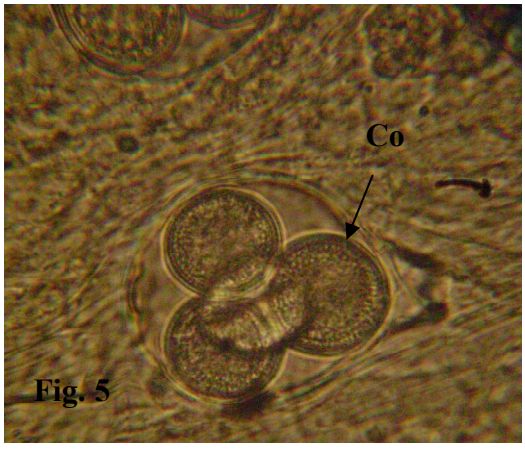


Fig. 4. *S. ferax* showing spherical pitted oogonia with centric oospores and no antheridial branches

Fig. 5. *S.Parasitica* showing spherical papillate oogonia with centric oospores and no antheridial branches.*PI, pitting, CO, centric oospore

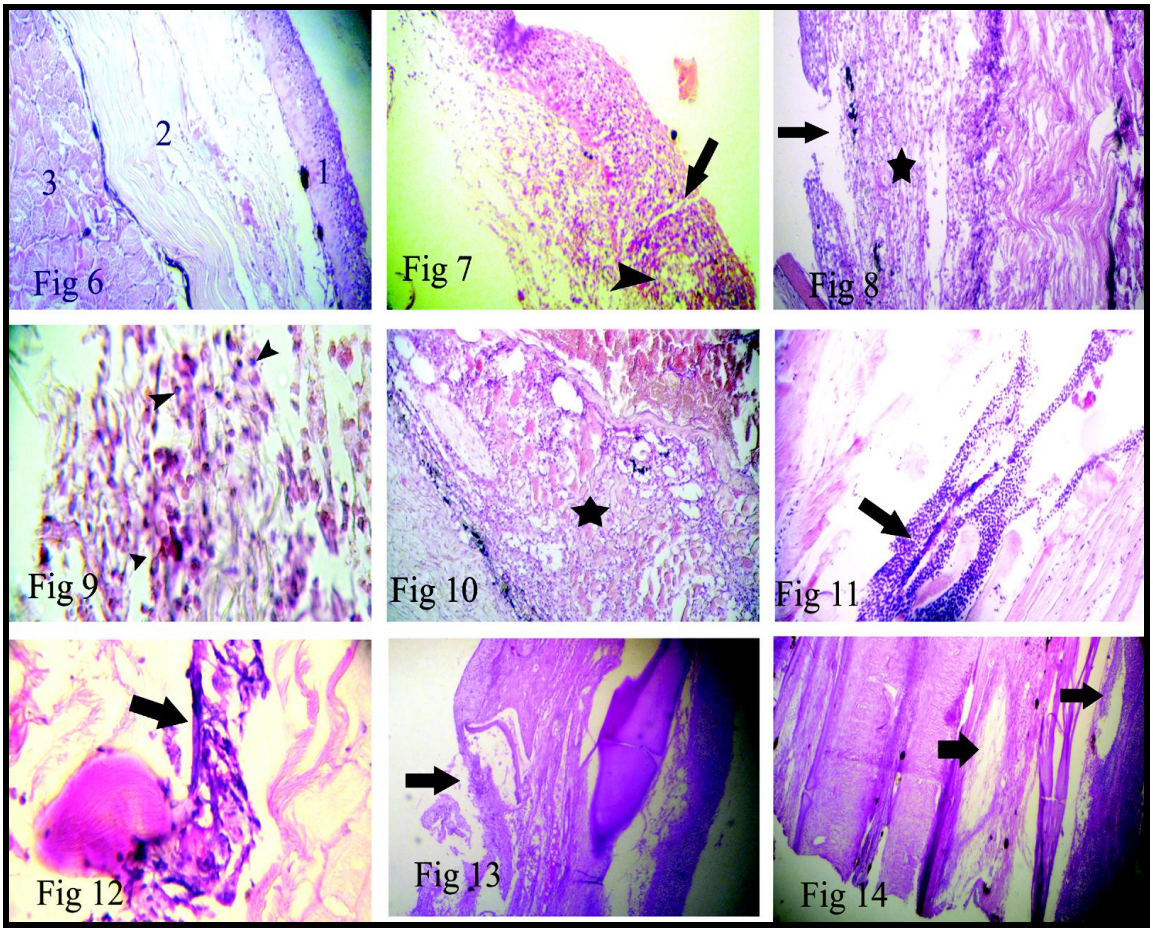


Fig. (6): Control Nile Tilapia. Normal skin consisting of epidermis 1 and dermis 2 then subcutaneous muscular tissue 3. HE 200x

Fig. (7): Infected Nile Tilapia. Epidermal cells are vacuolated (arrowhead) with the presence of minor erosion (arrow). HE 200x.

Fig. (8): Infected Nile Tilapia. Skin showing focal ulceration in the epidermis (arrow) with aggregation of inflammatory cells around spores and hyphae in superficial layer of dermis (star) HE 200x.

Fig. (9): Infected Nile Tilapia. High power to show aggregation of inflammatory cells in superficial layer of dermis (arrowheads). HE 200x

Fig. (10): Infected Nile Tilapia. Skin shows necrosis of subcutaneous muscles infiltrated with round cells (star) HE 200x

Fig. (11): Infected Nile Tilapia. Muscular tissue shows the presence of long aseptate basophilic hyphae surrounded by round cells (arrow). HE 200x

Fig. (12): Infected Nile Tilapia. Muscular tissue shows the presence of long aseptate basophilic hyphae (arrow). PAS 200x

Fig. (13): Infected Nile Tilapia. Caudal fin showing epidermal ulceration (arrow) HE 200x

Fig. (14): Infected Nile Tilapia. Caudal fin showing hypodermal edema (arrows) HE 200x

DISCUSSION

Nile Tilapia is one of the most dominant cultured fish in Egyptian fish farms. Due to environmental contamination and other risk stress factors; it can be prone to infection with subsequent huge economic losses. The infections in the present study were rapidly induced in comparison to others used a continuous spore challenge **(Howe and Stehly, 1998; Pottinger and Day, 1999).** This method induced severe uniform infections at the site of abrasion in all fish. The present study showed induction of saprolegniosis with two different isolates in Nile tilapia, the pathogenicity of the tested isolates was very high; however, the mortalities from *S. parasitica* were much higher than *S. ferax.* This can be explained as *S. parasitica* is one of the more destructive pathogenic isolates than *S. ferax* (**Van West, 2006).**

The higher cumulative mortalities resulted from both isolates of *saprolegnia* were in the same event reported by **Yuasa and Hatai (1995**) who rating the pathogenicity of juvenile rainbow trout with 15 Japanese isolates of S. parasitica as high (mortality between 93±100%), intermediate (mortality between 33±53%) and low or non-pathogenic (mortality between 0±9%). Moreover, the cumulative mortalities of the different salmonids fish groups exposed to $2x10^5$ spore/L concentrations of *S. salmonis* NJM 9851 were 90% for brown trout, 93.3% for sockeye salmon and 100% for rainbow trout, masu salmon, and Japanese char, however; all salmonid species exposed to $2x10^5$ spore/L concentrations of *S. parasitica* NJM 9868 had cumulative mortalities of 100**% (Hussein and Hatai, 2002**). In the same trend, Juvenile rainbow trout, *Oncorhynchus mykissn* (Walbaum), were experimentally infected with 20 isolates of two morphotypes of long-haired *Saprolegnia* using the "ami-momi" treatment. Pathogenicity varied greatly among isolates as mortality ranged from 0 to 100% of the fish **(Fregeneda Grandes et al., 2001)**. **Stueland et al., (2005)** found two of seven *Saprolegnia* spp. isolates tested for their pathogenicity to Atlantic salmon Salmo salar were significantly more pathogenic and caused 89% and 31% cumulative mortality in challenged salmonids than other isolates tested.

Lesions of *Saprolegnia* were in form of cottony like masses on different sites on fish body mainly in the dorsal region and on the dorsal and adipose fins **(Yanong, 2003)** which in same event with our results lesion appeared mostly in all sites which are exposed to scarification during descaling process. The death mainly occurred due to the osmotic failure caused by the damaged epidermis (**Bruno and Poppe, 1996).** The results of histopathological examination of skin, gills and fins of infected fish were similar to those observed by **(Amin et al., 1985, Ferguson, 1989, Aly and El Ashram, 2000, El Genaidy et al., 2004, Udomkusonsri and Noga 2005 and El Ashram et al., 2007). Fregeneda Grandes et al.**

(2001) found that *Saprolegnia* main histopathological lesions were presence of hyphae in the epidermis, dermis and subcutaneous muscle, in form of a large quantity of hyphae which replaced cellular content. Sloughing of epidermis was seen in some cases. In muscle, the hyphae invade through the connective tissue separating the bundles and muscle fibers, resulted in hyaline degeneration of these fibers. A common finding in all fishes challenged with *Saprolegnia* infection is that the main mass of invading mycelia start at the head region, adipose, dorsal and caudal fins, and that there is a lack of inflammatory response **(Wolke, 1975)**. Controversially, a severe inflammatory reaction was observed in all infected fish consisted of aggregation of round inflammatory cells in dermis and in subcutaneous muscular tissue. Agreeing with, **Noga et al., (1989)** who reported a cell-mediated response directed against mycelia that invade into deeper tissues beyond the epithelium. These inflammatory cells, called epithelioid cells, have epithelial features (desmosomes, tonofilaments). In the same trend, **Hussein and Hatai (2002)** reported same finding in experimental infection of salmonid species with different *Saprolegnia* isolates and were in form of loss of the epidermis, edema of the hypodermis and different degrees of degenerative changes in the underlying musculature. The cellular damage observed in the gills in terms of epithelial proliferation, separation of the epithelial layer from supportive tissues and necrosis can adversely affect the gas exchange and ionic regulation **(Dutta et al., 1993)**. The observed edematous changes in gill filaments and secondary lamellae probably due to increased capillary permeability **(Olurin et al., 2006).** The infection can apparently cause rapid destruction of the integument but the direct cause of death is probably related to impaired osmoregulation **(Hatai and Hoshiai, 1994 and Unestam, 1966)**. The extensive spread of edematous fluid between the muscle bundles was a characteristic finding in the infected fishes **(Hussein and Hatai, 2002**). The presence of myofibrillar degenerative changes in the muscle bundles some distance from the invading mycelial mat and the lack of toxins produced by *Saprolegnia* **(Nolard-Tintigner, 1973)** support the hypothesis that degenerative changes are partly due to the enzymatic activity of the invading mycelia **(Hussein and Hatai, 2002**). **Peduzzi and Bizzozero (1977)** demonstrated that the thalli of certain fish pathogenic isolates of *Saprolegnia* exhibit chymotrypsin-like activity, and claimed that this enzymatic activity is likely a contributing factor to the pathogenesis of saprolegniosis. Furthermore, PAS stain detected hyphae only in the subcutaneous muscle probably because of epidermal erosion and ulceration.

CONCLUSION

The present study has demonstrated that both isolates *S. parasitica* and *S. ferax* causing higher mortalities in Nile tilapia in Egypt, although the *S. parasitica* were found more pathogenic to Nile tilapia than the other species. Thus, presence of such pathogenic Oomycetes in Egyptian aquaculture imposes a high risk to healthy fish stocks.

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الملخص العربي

تأثير الضراوة التجريبية لمعزولتين من السابرولجنيا في أسماك البلطى النيلى في مصر مع إشارة خاصة إلى التغيرات النسيجية المرضيه

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الزغب القطنى الفطرى واحد من أهم الأمراض الفطرية التى تصيب أسماك المياه العذبه عالميا و تسبب خسائر اقتصادية هائلة. و لهذا فإن هذه الدراسة توضح معدلات النفوق فى أسماك البلطى النيلى الناتجه عن تأثير العدوى لاثنين من أهم معزولات السابرولجنيا وهى السابرولجنيا بارازيتيكا و السابرولجنيا فيراكس المعزولين من اندلاع طبيعى للسابرولجنيا في أسماك البلطى (أوريوكروميس نيلوتيكاس) في مزارع الأسماك المختلفه في مصر. تمت العدوى بمعزولتين هما السابرولجنيا بارازيتيكا و السابرولجنيا فيراكس بتركيز ٢×١٠[٤] من الحويصلات المتحركة /لتر لمدة ١٠ أيام. و كانت نسبة النفوق ٦.٥٩ ٪ و ٧٠ ٪ للسابرولجنيا بارازيتيكا و السابرولجنيا فيراكس بالترتيب. التغيرات النسيجية المرضيه قد تنوعت من تشكل فجوات، تآكل، تقرح الى الفقدان الكامل للطبق السطحية للجلد. كما أظهر باطن الجلد تورم و احتقان و تجمع لخلايا التهابية حول الخيوط الفطرية. العديد من التغيرات الانحلالية و النخرية لوحظت فى العضلات. أكدتالنتائج ليس فقط قدرة كلا من المعزولتين على إحداث المرض والنفوق و لكن وجد أيضا أن السابرولجنيا بارازيتيكا أكثر قدرة على إحداث معدل من النفوق فى أسماك البلطى النيلى أعلى من السابرولجنيا فيراكس.